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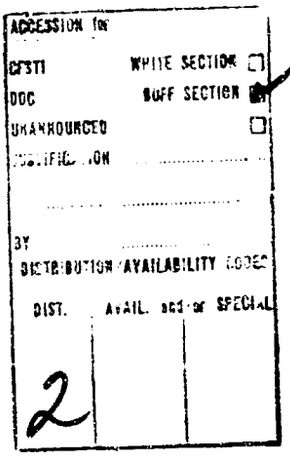
# PURIFICATION AND PHYSICAL PROPERTIES OF EASTERN EQUINE ENCEPHALITIS VIRUS

Anthony A. Fuscaldo
Edwin J. Hoffman
Halvor G. Aaslestad

APRIL 1970



DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland



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# PURIFICATION AND PHYSICAL PROPERTIES OF EASTERN EQUINE ENCEPHALITIS VIRUS

Anthony A. Fuscaldo

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Virus & Rickettsia Division
BIOLOGICAL SCIENCES LABORATORIES

Project 1B061102B71A01

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## ABSTRACT

A new purification procedure was adopted for eastern equine encephalitis virus that does not subject the virus to pelleting at any stage. Three- to four-liter volumes are passed through a DEAE cellulose column. The virus-containing fractions are banded on a sucrose cushion and finally banded isopycnally in a linear sucrose gradient. This method reduces the volume 1000-fold with a concomitant increase in viral titer. Numerous criteria were used to establish that this viral preparation is essentially free from cellular debris and nonviral material. Physical studies on this purified viral product were initiated. The sedimentation coefficient by the band sedimentation technique was 240 S. The calculated buoyant density in sucrose was 1.18 g/cc. The diameter of the virus was 54 mμ. From the diameter and the buoyant density it is possible to calculate the molecular weight of a spherical particle: for eastern equine encephalitis virus, it is 58 x $10^6$ daltons.

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## I. INTRODUCTION*

When determining the physical and biochemical properties of a virus particle, two prerequisites must be fulfilled. First, the virus preparation must be as free as is practicable of contaminating cellular and organic material; second, the purification and concentration steps should be as mild as possible to avoid procedurally induced damage to the virus particles. Another characteristic of the concentration and purification techniques should be the relative ease in handling large amounts of fluids containing virus.

We have found that pelleting the virus from tissue culture fluid does not meet at least two of these criteria. Either the pelleting or the resuspension steps probably cause some injury to the virus, as noted by both the loss in titer** and visible aggregation. Furthermore, the pelleting procedure becomes difficult with volumes greater than 1 liter.

For these reasons, we have adapted the DEAE cellulose column purification technique of Fraser*** to animal virus systems.

## II. MATERIALS AND METHODS

### A. VIRUS AND CELL CULTURE

The Louisiana strain of eastern equine encephalitis (EEE) virus, the origin and properties of which were reported by Brown[1] was used in all experiments. All the virus was propagated in 60-mm petri dishes by washing 24-hour CE monolayers with Earle's saline A[2] and infecting with $5 \times 10^6$ to $1 \times 10^7$ pfu of a mouse brain suspension of EEE. The virus was allowed to adsorb for 30 minutes at room temperature, excess inoculum was removed, and then plates were overlayed with 2.5 ml of lactalbumin hydrolysate plus 10% calf serum.[3] The tissue culture was harvested after 16 hours at 37 C in a 5% $CO_2$ atmosphere, and the plates were then fed with another 2.5 ml of the same medium. Four to six hours later, a second harvest was made. The virus titer was assayed using the plaque technique described by Zebovitz and Brown.[4] The titer of the first harvest was usually around $5 \times 10^9$ to $1 \times 10^{10}$ pfu/ml and that of the second between $2 \times 10^9$ and $4 \times 10^9$. The crude viral harvests were then stored at -70 C.

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* This report should not be used as a literature citation in material to be published in the open literature. Readers interested in referencing the information contained herein should contact the senior author to ascertain when and where it may appear in citable form.

** Personal communication, Fred P. Heydrick, Fort Detrick.

*** Personal communication (manuscript in preparation), W.D. Fraser, Department of Microbiology, Indiana University, Bloomington, Indiana.

## B. PURIFICATION

The general purification scheme is shown in Figure 1. The crude viral harvests were dialyzed overnight against four to five volumes of distilled water. Three to four liters of the dialyzed material was then applied to a DEAE-cellulose column. The column was prerun at least once and then washed with 0.05 M NaCl before use in the purification. The column can be reused numerous times, finally being discarded when the flow falls below 10 ml/min. After washing the column with 0.15 M NaCl, the virus was eluted with 0.7 M Tris succinate saline (TSS) buffer,* pH 9.0, in 20- to 25-ml fractions. The virus-containing fractions were identified by their light-scattering properties.

Aliquots (25 ml) of these fractions were then layered onto a 5-ml sucrose cushion (45% sucrose) and centrifuged for 2.5 hours at 78,500 x g in the Spinco Model L ultracentrifuge.

The visible virus band was collected, diluted 1:2 with a glycine-NaOH-KCl buffer**(pH 9.0), and layered onto a 30 to 50% sucrose gradient. This was centrifuged to equilibrium for 20 hours at 62,000 x g in the Spinco Model L ultracentrifuge. The virus band was collected by a side puncture of the tube and was stored in the buffered sucrose at 4 C. The virus maintains viability for one to two weeks under these conditions.

## C. ELECTRON MICROSCOPY

Samples were negatively stained with phosphotungstic acid (PTA). Some were also shadowed with uranium. All work was done on an RCA EMU-4 electron microscope.

## D. SEDIMENTATION STUDIES

The S value was obtained by band sedimentation, using Studier's[5] adaptation of the Vinograd technique. The Spinco Model E analytical ultracentrifuge equipped with UV adsorption optics was used.

## E. BUOYANT DENSITY

Buoyant density studies were done in 30 to 50% linear sucrose gradients centrifuged 24 hours to equilibrium. Fractions were collected from a hole punched in the bottom of the tube. All of the fractions were assayed for infectivity, and every other fraction also was read on an Abbe refractometer for its density.

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* Personal communication, W.D. Fraser.

** 0.05 M glycine, $8.8 \times 10^{-3}$ M NaOH, 0.1 M KCl.

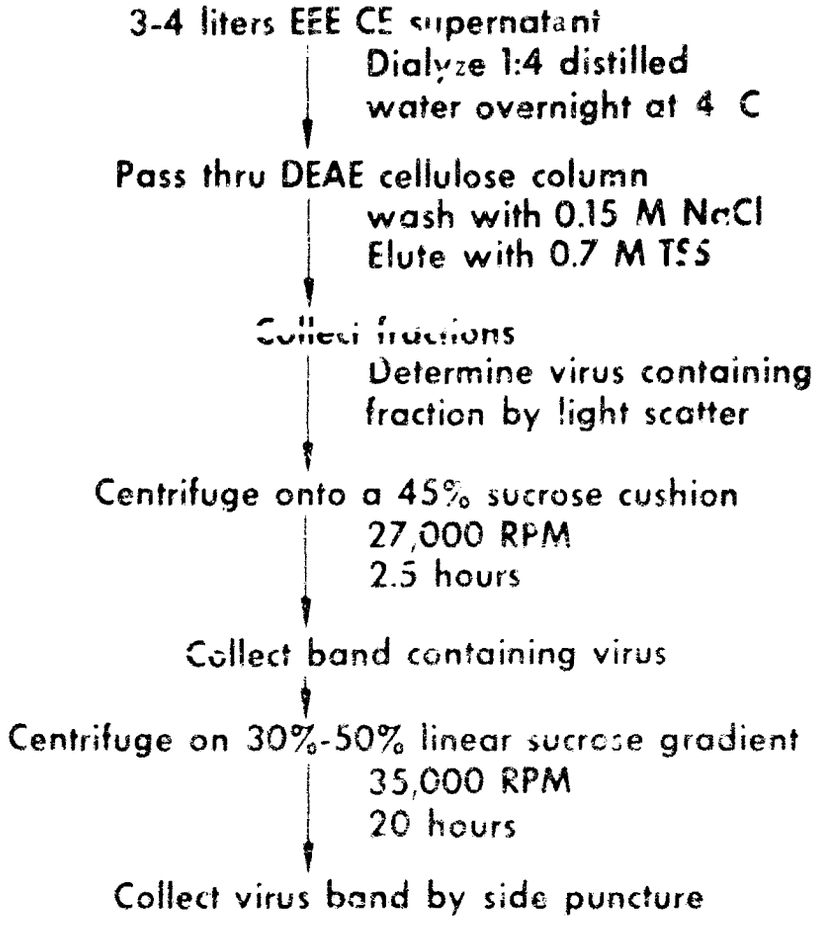


FIGURE 1. General Purification Scheme for EEE Virus.

## F. RADIOLOGICAL METHODS

CE cells were labeled by adding $^{14}C$ amino acids* and $^{32}PO_4$** to the growth medium. After 20 hours, the medium was removed from the wall of the T-60 flask, and the cells were washed, removed, and sonicated. The radioactive cell debris was added to the "cold" viral harvest prior to purification. Aliquots (0.1 ml) of the starting material and of every fraction taken thereafter were placed on Whatman No. 1 filters. The dried filters were counted in toluene-based BBOT*** with a Nuclear Chicago or a Packard liquid scintillation spectrometer. The data curve was corrected for spillover ($^{32}P$ counts in the $^{14}C$ channel and the reverse were applicable) with a Remington-Olivetti desk-top computer.

# III. RESULTS

## A. PURIFICATION

In order to monitor the efficiency of the purification procedure, the following reconstruction experiment was undertaken. Host cellular material was labeled with $^{14}C$ amino acids and $^{32}PO_4$. The cells were sonicated, and the resultant $^{14}C$-labeled proteins, $^{32}P$-labeled RNA, DNA, and phospholipid were added to unlabeled virus that was to be purified. The fate of cellular contaminants in the viral preparation was then monitored by the isotope dilution technique. Figure 2 shows the distribution of radioactivity after passing the material through the DEAE-cellulose column. Approximately 55% of the total radioactivity is associated with the infectivity peak, of which about 40% is contained in tubes 21 to 24. Of the remaining 45% of the total radioactivity, 25% is associated with the void volume, while 20% remains tightly bound to the column. The radioactivity associated with the virus product is further diminished by the sucrose cushion step to 0.1% of the original activity. Figure 3 shows the results of the isopycnic sucrose gradient. As this figure illustrates, the final isopycnally banded virus product contains practically none of the radioactive contaminant.

Another indication of the purity of the viral preparation is its optical density (OD) profile. Figure 4 shows these profiles for material taken from the sucrose cushion and from the sucrose gradient. The sucrose cushion material has an adsorption maxima at 270 mμ, indicating that there is still a significant amount of protein associated with the viral material. However, the profile of the material taken from the isopycnic sucrose gradient shows that the maxima has shifted to 262 mμ. The latter OD profile, similar to the profile of viral RNA, would be expected for a virus containing approximately 5% RNA and 70% protein.

---

* Schwarz BioResearch, Inc., Orangeburg, N.Y.

** Abbott Laboratories, Baltimore, Md.

*** 2,5-bis[2-(5-tert-butylbenzoxazolyl)]-thiophene.

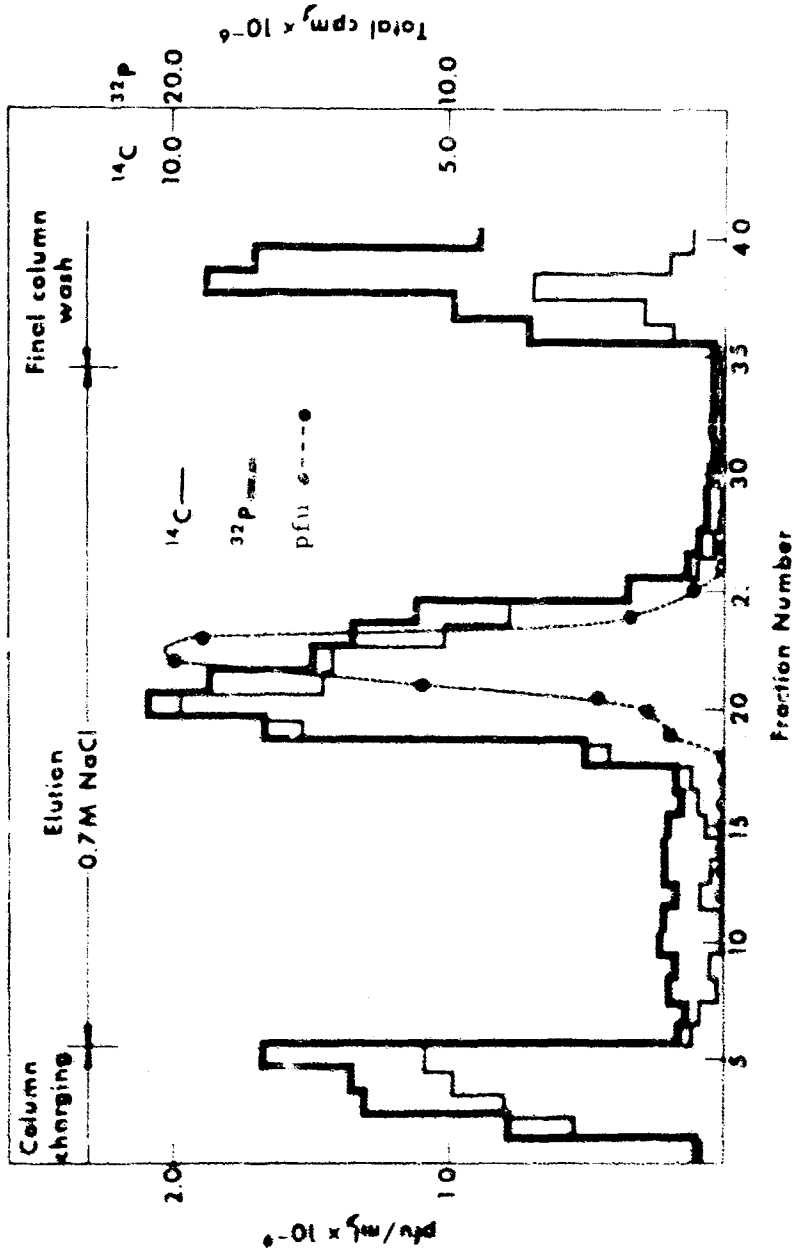


FIGURE 2. Distribution of EEE Virus Plaque-Forming Units and Radioactive Cellular Debris after Passage through a DEAE-Cellulose Column.

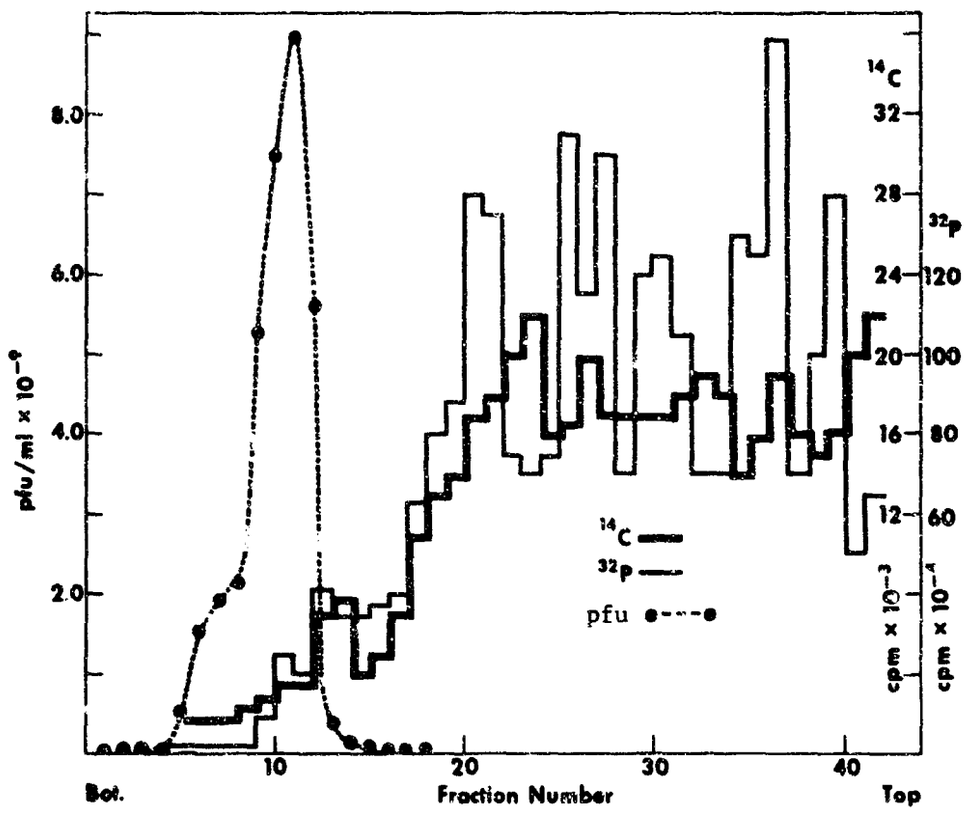


FIGURE 3. Distribution of EEE Virus Plaque-Forming Units and Radioactive Cellular Debris after Isopycnic Sucrose Gradient Centrifugation.

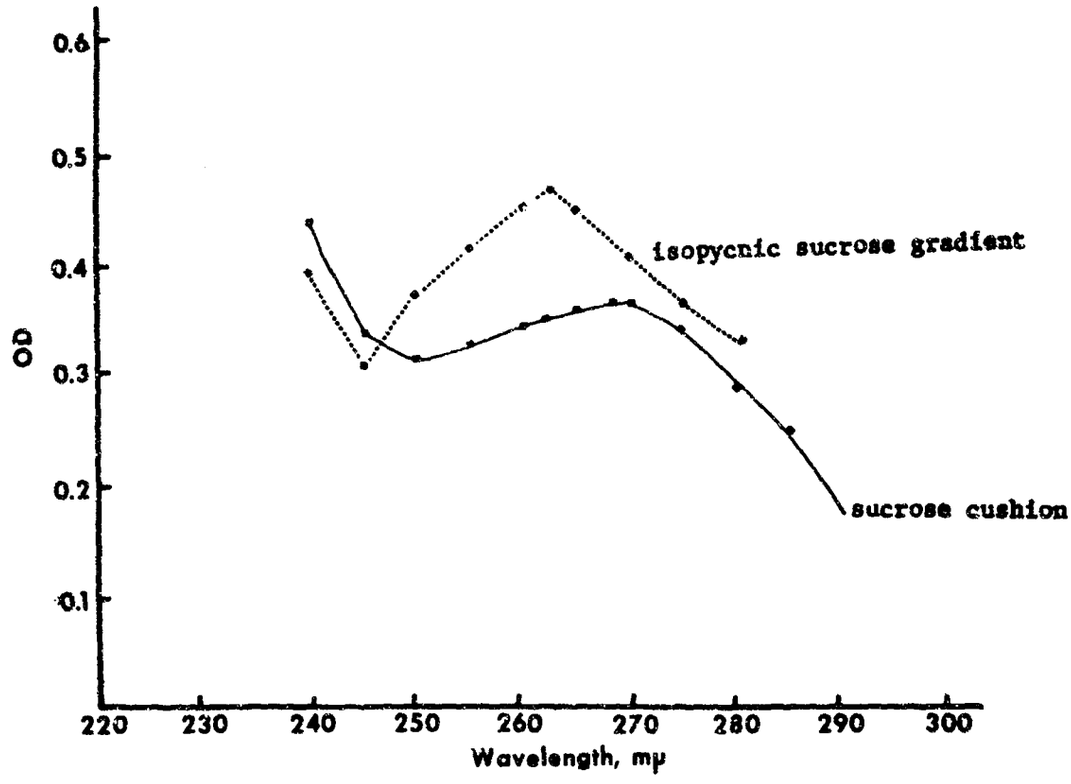


FIGURE 4. Optical Density Profiles of EEE Virus Material after Each of the Last Two Purification Steps.

The overall efficiency of the purification scheme is summarized in Table 1. The combined concentration effect shows a volume reduction of 500-fold, which in turn increases the infectivity to over 10,000 times that of the starting material. The per cent recovery of pfu is greater than 100% at the final step, which is probably due to an increased efficiency of plating (EOP). The actual increase in total virus is 10-fold. This increase in total virus can be duplicated by sonication of the virus-containing tissue culture fluid. Therefore, it seems as if the purification process is disrupting large clumps of virus in addition to purifying and concentrating the virus. Also included in the table is the result of the isotope dilution experiment. Finally, there is the ratio of pfu to protein at each step of the procedure. The final ratio, $5.0 \times 10^{12}$, is within a factor of three of the theoretical maximum that would be calculated on the basis of every particle being infectious. This calculation is made by utilizing a particle weight of $58 \times 10^6$, which is determined in a later section of this paper, and a protein contribution of 72% of the intact virus.* These data lead to the assumption that one particle in three of our purified preparation is infectious. This assumption is being investigated by relating total particles per ml as determined by electron microscopy to pfu per ml.

TABLE 1. EFFICIENCY OF PURIFICATION OF EEE VIRUS BY THE DEAE-CELLULOSE ELUTION METHOD

| | Crude Tissue Culture Medium | Column Eluate | Sucrose Cushion | Purified Product |
|---|---|---|---|---|
| Titer, pfu/ml | $3.0 \times 10^9$ | $1.6 \times 10^{11}$ | $1.5 \times 10^{13}$ | $1.9 \times 10^{13}$ |
| Volume, ml | 3,300 | 69 | 4 | 6 |
| Recovery of pfu, % | - | 100 | >100 | >100 |
| Labeled cell debris, % | 100 | 35 | 0.1 | 0.03 |
| Protein, pfu/mg | $3.0 \times 10^8$ | $2.7 \times 10^{10}$ | $1.1 \times 10^{12}$ | $5.0 \times 10^{12}$ |

## B. PHYSICAL STUDIES ON THE VIRUS PARTICLE

The sedimentation coefficient of the virus particle was determined by the band sedimentation technique.[5] The S value was 240. This figure is the $S^\circ_{20}$, but it was not corrected for viscosity and density of the medium. The tracings of a typical experiment also show only one peak (Fig. 5). The slight skewing of the band to the leading edge is a characteristic of band sedimentation experiments.

* A.A. Fuscaldo, F.P. Heydrick, and E.J. Hoffman, unpublished data.

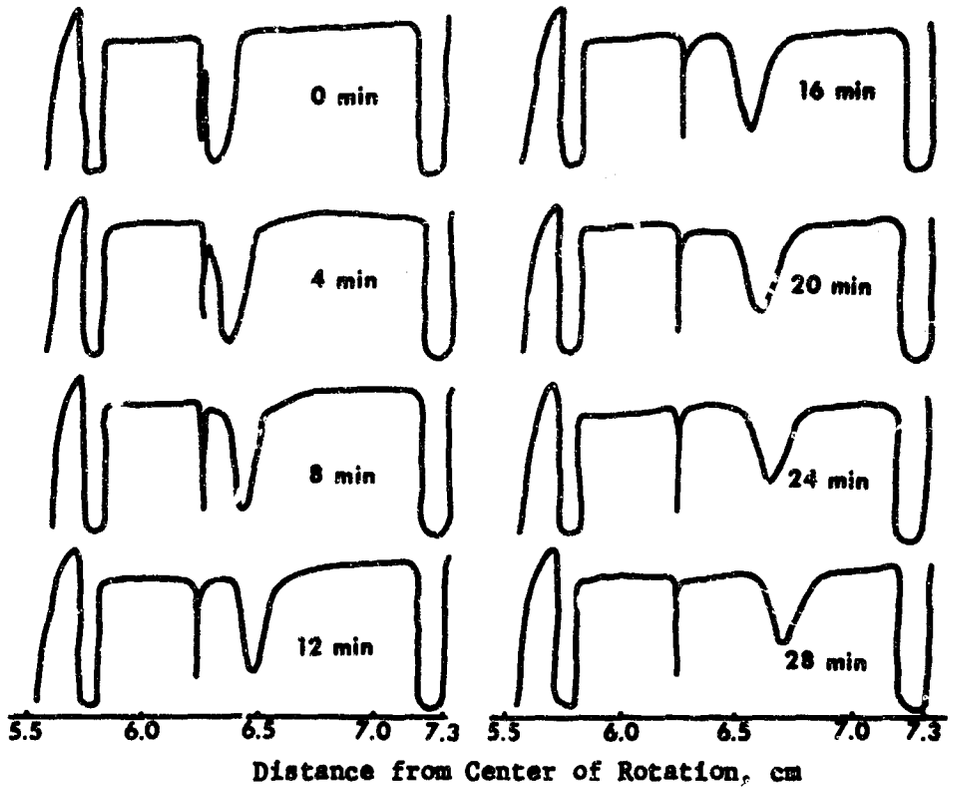


FIGURE 5. Typical Band Sedimentation Tracings of EEE Virus Purified by the DEAE-Cellulose Method.

The buoyant density of the virus was determined in a linear sucrose gradient (30 to 50%) that was centrifuged for 20 hours at 63,500 x g in the Spinco Model L ultracentrifuge. Figure 6 gives the results of this experiment, which showed that the buoyant density of EEE is 1.18.

A negatively stained electron micrograph of the intact virus is shown in Figure 7. It was determined from the measurement of a large number of particles in numerous electronmicrographs that this is a spherical virus with a diameter of 54±5 mμ.

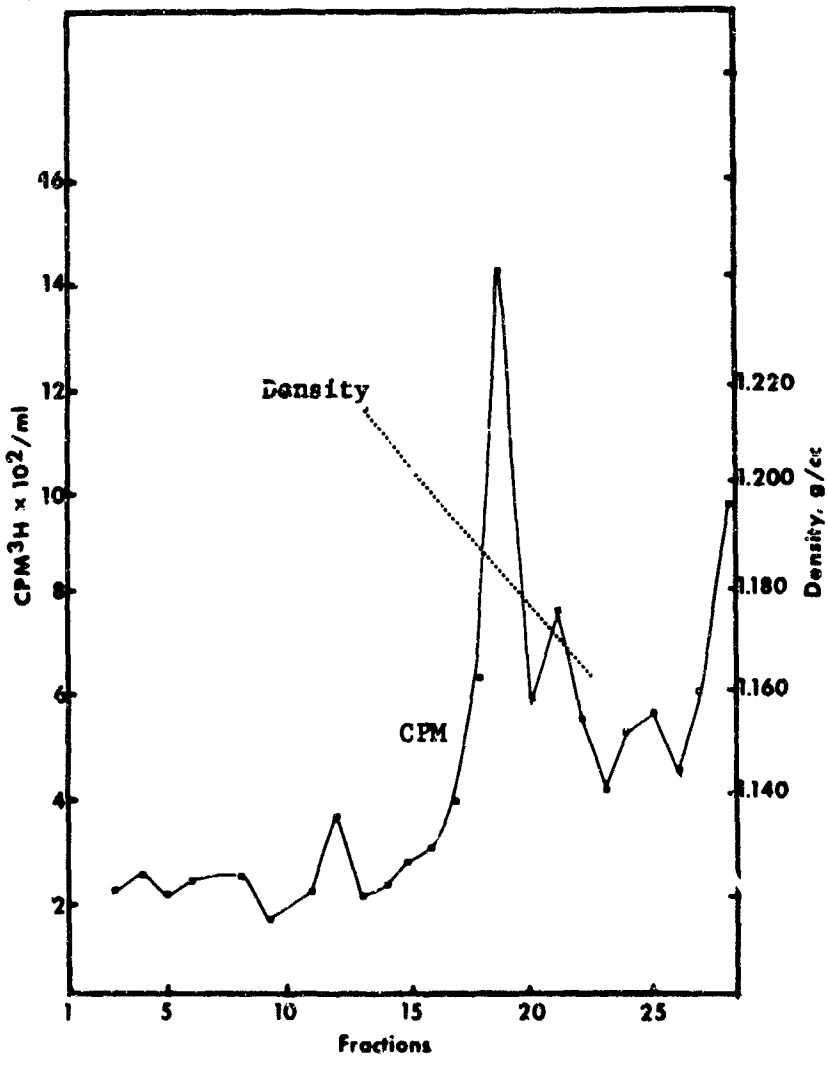


FIGURE 6. Bouyant Density of Purified EEE Virus in Sucrose.

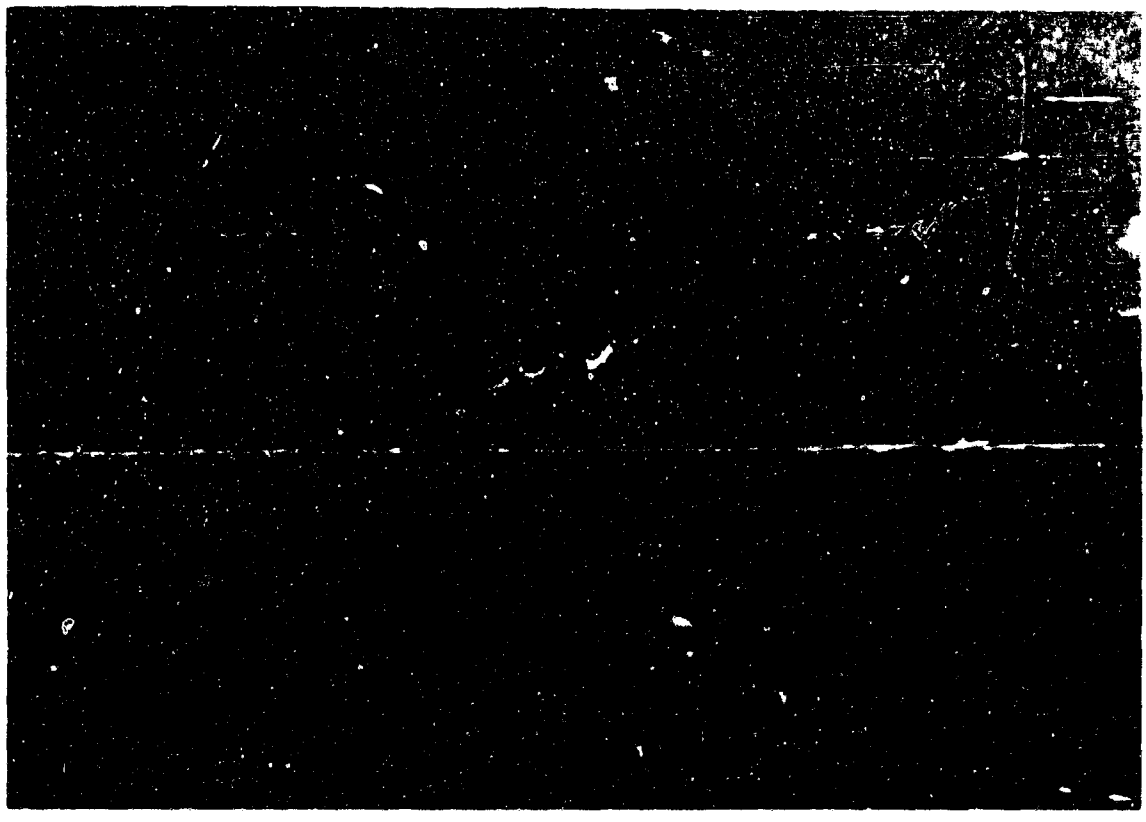

FIGURE 7. Typical Negatively Stained Electron Micrograph of Purified Intact EEE Virus.

## IV. DISCUSSION

We feel that the virus purification technique utilized here is both simple and highly efficient. The separation of extraneous debris from the virions is shown by the results in Figures 2, 3, and 4 and in Table 1.

The shift in the OD maximum shown in Figure 4 would be expected with the removal of nonviral protein from the virus preparation. A virus particle that contains approximately 5% nucleic acid, 75% protein and 20% lipid* would be expected to have an OD maximum of 258 to 262 mμ because RNA has a much greater extinction coefficient than protein. The removal of nonviral protein is also indicated by the greatly increased pfu:protein ratio (Table 1).

Work on the physical characteristics of this virus poses some difficulties. We were able to determine an S value for the virus at 20 C and also extrapolated back to zero concentration of virus. However, correction of the value for the solvent to make it comparable to the value for centrifugation through water presents a problem. It is necessary to assume a partial specific volume, $\bar{v}$, for the virus, and this cannot be done with any reasonable accuracy for a particle as complex as an arbovirus. Attempting to calculate a particle weight from sedimentation data is further complicated by the fact that the $\bar{v}$ is needed again for that calculation.

The buoyant density calculated for this virus, 1.18, is lower than that obtained by Aaslestad, Hoffman, and Brown.[6] However, their experiments were carried out in CsCl, while ours were performed in a linear sucrose gradient. Their work also yielded three bands, indicating that the virus particles were at least partially disrupted. Other workers[7,8] have also noted that viruses containing lipid have slightly lighter densities in sucrose than in CsCl. This difference usually is attributed to differing effects of hydration in the two solutions. However, we have carried out experiments in which the virus buoyant density is determined in sucrose, then subsequently layered on CsCl, and the density determined in this solution. The band containing most of the infectivity is then rerun on sucrose and the buoyant density determined for the third time. A control was also run through three sucrose gradients. The results indicated that the difference is due to partial disruption of the virus envelope by the CsCl.

Because a determination of particle weight from the $S^{0}_{w}$ 20 value requires an accurate estimate of $\theta$, we felt it impossible to make that calculation at this time. For this reason, we attempted to determine the particle weight of the virus by utilizing the volume of a spherical particle and the experimentally determined density of the virus particle. The following formula was used:

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* A.A. Fuscaldo, F.P. Haydrick, and E.J. Hoffman, unpublished data.

$$M = \frac{N\pi D^3 \rho}{6}$$

where N = Avogadro's number, D = diameter of the virus measured in cm, and $\rho$ = the density of the virus. A basic difficulty with this formula is the assumption that the buoyant density can be substituted for the actual density. We have made this assumption on the basis that we can substitute the buoyant density into another formula and derive an S value that approximates the experimentally determined one. The formula used was derived directly from Stokes' law for the coefficient of friction for a spherical body, assuring a dilute, ideal solution, negligible diffusion, and a spherical body. The formula was:

$$S = \frac{(\rho v - \rho m)\frac{1}{2}D}{0.426}$$

where $\rho v$ = density of the virus, $\rho m$ = density of the medium, and D = diameter of the particle in cm. Through the use of the above formulae we have determined that the EEE virus particle has a molecular weight of $58 \times 10^6$ and the calculated S value is 227 compared with an experimentally determined value of 240 S. The calculated S value was within 10% of the uncorrected, experimental S value obtained. Since the correction of the S value for a spherical particle would most likely be in the order of 10% or less, we feel justified in our original assumption concerning the use of buoyant density. Furthermore, when using these formulae, close approximation to the literature values for particle weights and S values can be attained.

Now that EEE virus has been purified and concentrated to a high degree, it is possible to make a detailed analysis of the chemical composition and structure of the components. Furthermore, knowledge of these physical parameters of the intact virion should be of assistance in determining both the subparticle structure and the morphogenesis of the virus particle.

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13. ABSTRACT

A new purification procedure was adopted for eastern equine encephalitis virus that does not subject the virus to pelleting at any stage. Three- to four-liter volumes are passed through a DEAE cellulose column. The virus-containing fractions are banded on a sucrose cushion and finally banded isopycnally in a linear sucrose gradient. This method reduces the volume 1000-fold with a concomitant increase in viral titer. Numerous criteria were used to establish that this viral preparation is essentially free from cellular debris and nonviral material. Physical studies on this purified viral product were initiated. The sedimentation coefficient by the band sedimentation technique was 240 S. The calculated buoyant density in sucrose was 1.18 g/cc. The diameter of the virus was 54 mμ. From the diameter and the buoyant density it is possible to calculate the molecular weight of a spherical particle: for eastern equine encephalitis virus, it is 58 x $10^6$ daltons.

14. Key Words

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DEAE cellulose column
Sucrose cushion
Sucrose gradient

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